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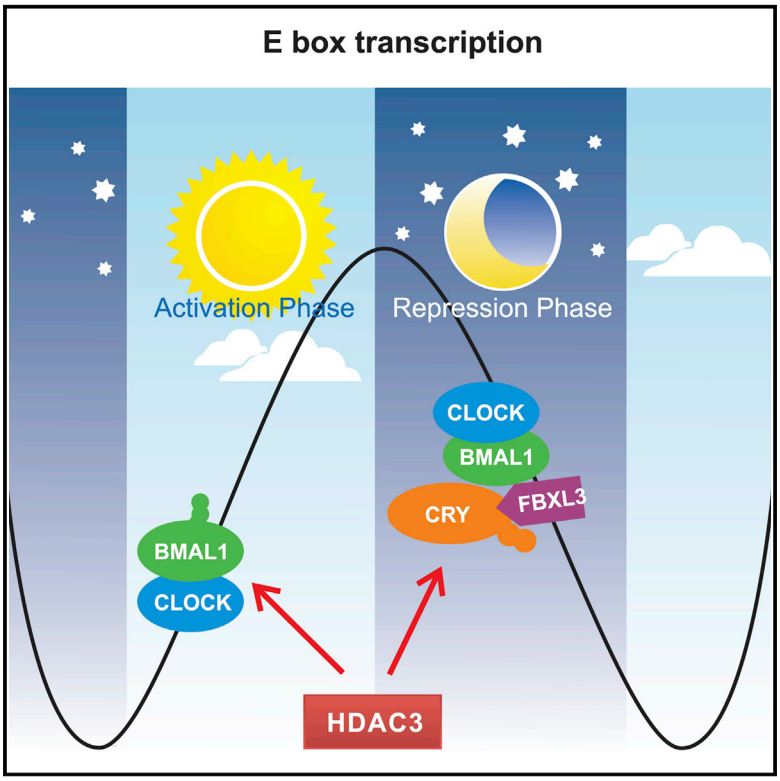
Cell Reports

# Distinct Roles of HDAC3 in the Core Circadian Negative Feedback Loop Are Critical for Clock Function

## Graphical Abstract

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## In Brief

Shi et al. report a deacetylase-independent function of HDAC3 in maintaining a robust circadian clock. HDAC3 modulates the function of several key components of the clock machinery and affects both the activation and suppression of transcription in the circadian cycle.

## Highlights

- HDAC3 is a critical component of the core mammalian circadian negative feedback loop
- HDAC3 is required for efficient activation of E-box-driven gene expression
- HDAC3 blocks CRY1 degradation and promotes BMAL1 and CRY1 association
- Temporal separation of these opposing roles ensures robust circadian gene expression

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# Distinct Roles of HDAC3 in the Core Circadian Negative Feedback Loop Are Critical for Clock Function

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## SUMMARY

**In the core mammalian circadian negative feedback loop, the BMAL1-CLOCK complex activates the transcription of the genes *Period* (*Per*) and *Cryptochrome* (*Cry*). To close the negative feedback loop, the PER-CRY complex interacts with the BMAL1-CLOCK complex to repress its activity. These two processes are separated temporally to ensure clock function. Here, we show that histone deacetylase 3 (HDAC3) is a critical component of the circadian negative feedback loop by regulating both the activation and repression processes in a deacetylase activity-independent manner. Genetic depletion of *Hdac3* results in low-amplitude circadian rhythms and dampened E-box-driven transcription. In subjective morning, HDAC3 is required for the efficient transcriptional activation process by regulating BMAL1 stability. In subjective night, however, HDAC3 blocks FBXL3-mediated CRY1 degradation and strongly promotes BMAL1 and CRY1 association. Therefore, these two opposing but temporally separated roles of HDAC3 in the negative feedback loop provide a mechanism for robust circadian gene expression.**

## INTRODUCTION

Eukaryotic circadian clocks consist of autoregulatory feedback loops that regulate a wide variety of molecular and physiological activities (Bass and Takahashi, 2010; Partch et al., 2014). In the core mammalian circadian negative feedback loop, the BMAL1-CLOCK complex is the positive element that activates the transcription of *Period* (*Per*), *Cryptochrome* (*Cry*), and clock-controlled genes by binding to E-boxes in their promoters. In contrast, the PER-CRY complex is the negative element that closes the negative feedback loop by repressing the activity of the BMAL1-CLOCK complex (Hardin and Panda, 2013; Lowrey and Takahashi, 2004; Reppert and Weaver, 2002; Schibler and Naef, 2005). The cyclic activation and repression of E-box-driven transcription is thought to be the major basis for endogenous circadian rhythmicity in mammals (Lowrey and Takahashi, 2011).

BMAL1 is essential for circadian rhythm generation and is subject to multiple forms of post-translational modifications that are important for its function (Bunger et al., 2000). BMAL1 is a substrate for sumoylation, and this modification is essential for circadian clock oscillation (Cardone et al., 2005). More recently, it has been shown that BMAL1 is also ubiquitylated and that BMAL1-CLOCK activity is coupled tightly with the level of BMAL1 ubiquitylation. First, transcriptional activation by BMAL1-CLOCK correlates with high BMAL1 ubiquitylation levels (Lee et al., 2008). Second, BMAL1-CLOCK, in association with the E-box-containing *Dbp* promoter, is very unstable and shows a proteasome-dependent fluctuation (Stratmann et al., 2012). In addition, BMAL1 exhibits a robust circadian rhythm in phosphorylation profiles (Tamaru et al., 2003, 2009), and it can be acetylated by CLOCK and de-acetylated by the nicotinamide adenine dinucleotide (NAD)+-dependent SIRT1 enzyme (Hirayama et al., 2007; Nakahata et al., 2008).

The repression of the BMAL1-CLOCK complex by PER-CRY is mediated by their interaction (Kiyohara et al., 2006; Nangle et al., 2014; Partch et al., 2014; Ye et al., 2011), which peaks in the subjective night around circadian time (CT) 15–18 (Lee et al., 2001). In the PER-CRY complex, CRYs are thought to be the major repressors (Griffin et al., 1999; Kume et al., 1999; Langmesser et al., 2008; Ye et al., 2014). Although PER has been shown to promote the BMAL1-CRY interaction (Chen et al., 2009), the potent ability of CRY1 to repress BMAL1-CLOCK-induced transcription in the absence of PERs was observed under different systems (Partch et al., 2014; Shearman et al., 2000; Ye et al., 2014). Recent studies have shown that PER may compete with

FBXL3 to bind CRYs and, therefore, stabilize CRYs (Nangle et al., 2014; Xing et al., 2013), and PER may also counteract CRY function by maintaining CLOCK phosphorylation (Matsumura et al., 2014). These findings suggest that PER proteins have multiple effects on the circadian clock and CRYs (Brown et al., 2005; Duong et al., 2011; Duong and Weitz, 2014; Koike et al., 2012; Miki et al., 2012; Padmanabhan et al., 2012). CRYs are ubiquitylated by two competing E3 ligase complexes containing the substrate receptors FBXL3 and FBXL21 and are degraded by the proteasome pathway (Busino et al., 2007; Hirano et al., 2013; Siepka et al., 2007; Xing et al., 2013; Yoo et al., 2013). The degradation of CRY allows the reactivation of the BMAL1-CLOCK complex and the re-initiation of CRY and PER transcription in the subjective morning.

In addition to the core negative feedback, a secondary feedback loop consists of the orphan nuclear receptors REV-ERBα/β and retinoid-related orphan receptor (RORs), which act on the Rev-Erb/ROR-binding element (RREs) to regulate *Bmal1* and *Cry1* expression (Preitner et al., 2002; Sato et al., 2004; Ueda et al., 2005; Ukai-Tadenuma et al., 2011). REV-ERBα/β recruits the nuclear receptor corepressor (N-CoR) and histone deacetylase 3 (HDAC3) to regulate homeostasis between circadian clock and metabolic pathways (Alenghat et al., 2008; Yin and Lazar, 2005). HDAC3 is recruited by REV-ERBα to direct genome-wide rhythmic histone acetylation and to mediate circadian clock output pathways (Feng et al., 2011; Yin and Lazar, 2005). We found recently that deletion of *Fbxl3* impairs the REV-ERBα-HDAC3-mediated suppression of *Bmal1* and *Cry1* transcription (Shi et al., 2013). Although HDAC3 is primarily known to function as a deacetylase, and the crystal structure of HDAC3 and its co-repressor N-CoR2 suggests that HDAC3 also likely serves to stabilize protein-protein interactions (Watson et al., 2012). Consistent with this model, a deacetylase-independent function of HDAC3 in transcription and metabolism has been reported recently (Sun et al., 2013).

Here we demonstrate that HDAC3, in addition to regulating rhythmic transcription via histone deacetylation, is a critical component of the core circadian negative feedback loop independent of its deacetylase activity. The genetic depletion of HDAC3 results in low-amplitude circadian rhythms of E-box-driven genes. During the subjective morning, HDAC3 promotes BMAL1-dependent transcriptional activation. To close the negative feedback loop, HDAC3 facilitates the association between BMAL1 and CRY1 during subjective night. Therefore, these two temporally separate roles of HDAC3 act on both positive and negative processes of the negative feedback loop to achieve robust circadian gene expression.

## RESULTS

### Genetic Depletion of *Hdac3* Severely Reduces the Amplitude of Circadian Rhythms

To understand the role of *Hdac3* in the mammalian circadian clock, we generated a conditional knockout mouse line termed *Hdac3*$^{tm1a(EUCOMM)Wtsi}$ (*Hdac3*$^{f/f}$) using the European Conditional Mouse Mutagenesis Program (EUCOMM) Embryonic Stem Cell Resource (Figure 1A). Because whole-body deletion of *Hdac3* results in very early embryonic lethality (Bhaskara et al., 2008), *Alb-Cre* (*Cre* recombinase transcription under the control of the *Albumin* promoter) mice were crossed with *Hdac3*$^{f/f}$ mice to generate liver-specific *Hdac3* knockout mice (*Alb*; *Hdac3*$^{f/f}$) (Knutson et al., 2008). *Alb-Cre* is expressed in parenchymal liver cells, resulting in roughly 40% recombination hepatocytes at birth and almost complete recombination by 2 weeks after birth (Postic and Magnuson, 2000). Western blot analysis revealed that the expression of HDAC3 was reduced markedly but not abolished completely in the liver lysate from the *Alb*; *Hdac3*$^{f/f}$ mice, potentially because of non-hepatocyte cells in the liver tissue (Figure 1B).

To determine the role of HDAC3 in clock function, we crossed *Alb*; *Hdac3*$^{f/-}$ mice to *Hdac3*$^{f/f}$; *mPER2*$^{Luc}$ knockin reporter mice (Yoo et al., 2004) and monitored *Per2*-luciferase oscillation in liver tissues. As shown in Figure 1C, in contrast to the robust circadian bioluminescence rhythms in control *Hdac3*$^{f/f}$ mouse liver tissues, the *Alb*; *Hdac3*$^{f/f}$ liver displayed an apparent lower amplitude in bioluminescence rhythms. The normalized amplitude of *Alb*; *Hdac3*$^{f/f}$ strain was significantly lower than that of *Hdac3*$^{f/f}$ mouse liver from three independent experiments. This result indicates that the reduction of HDAC3 expression in the liver severely affected clock function. In contrast, normal circadian bioluminescence rhythms were observed in lung and suprachiasmatic nucleus (SCN) explants of *Alb*; *Hdac3*$^{f/f}$ mice, in which *Hdac3* expression is present (Figure S1).

To further confirm the role of HDAC3, we constitutively overexpressed HDAC3 or GFP in mouse embryonic fibroblasts (MEFs) isolated from *mPER2*$^{Luc}$ embryonic day (E) 13.5–E14.5 mouse embryos by infecting the cells with recombinant adenovirus expressing HDAC3 (Ad-HDAC3) or GFP (Ad-GFP) under control of the cytomegalovirus (CMV) promoter (Yoo et al., 2004). Western blot analysis of whole-cell extracts showed that infection with the Ad-HDAC3 virus resulted in elevated HDAC3 levels (Figure 1D, right). The constitutive elevation of *Hdac3* expression in the *mPER2*$^{Luc}$ MEFs increased the amplitude of the bioluminescence oscillation of *Per2*-luciferase (Figure 1D). Together, these results indicate that HDAC3 plays a critical role in maintaining high-amplitude circadian gene expression.

### Depletion of *Hdac3* Affects Circadian Gene Expression and Results in Low BMAL1 and CRY Protein Levels

We then examined the levels of clock gene expression in *Alb*; *Hdac3*$^{f/f}$ liver tissues. RNA was prepared at various circadian time points in the *Hdac3*$^{f/f}$ (hereafter referred to as wild-type [WT]) and *Alb*; *Hdac3*$^{f/f}$ (knockout) liver tissues. As shown in Figure 2A, depletion of *Hdac3* resulted in significant reductions in the peak transcript levels of E-box-driven genes such as *Per1*, *Per2*, *Cry2*, and *Dbp* (Figure 2A). Although *Cry1* transcription is driven by E-box, which should be reduced by the depletion of *Hdac3*, such an effect can be counterbalanced by the derepression of RRE-driven transcription. As a result, *Cry1* mRNA levels were not affected by the depletion of *Hdac3*. The N-CoR/HDAC3 complex has been shown previously to be recruited by REV-ERBα to repress RRE-driven gene transcription (Yin and Lazar, 2005). However, knockout of *Hdac3* does not completely phenocopy the *Rev-Erbα* mutant, suggesting that *Rev-Erbα* might have other functions in addition to recruiting HDAC3.

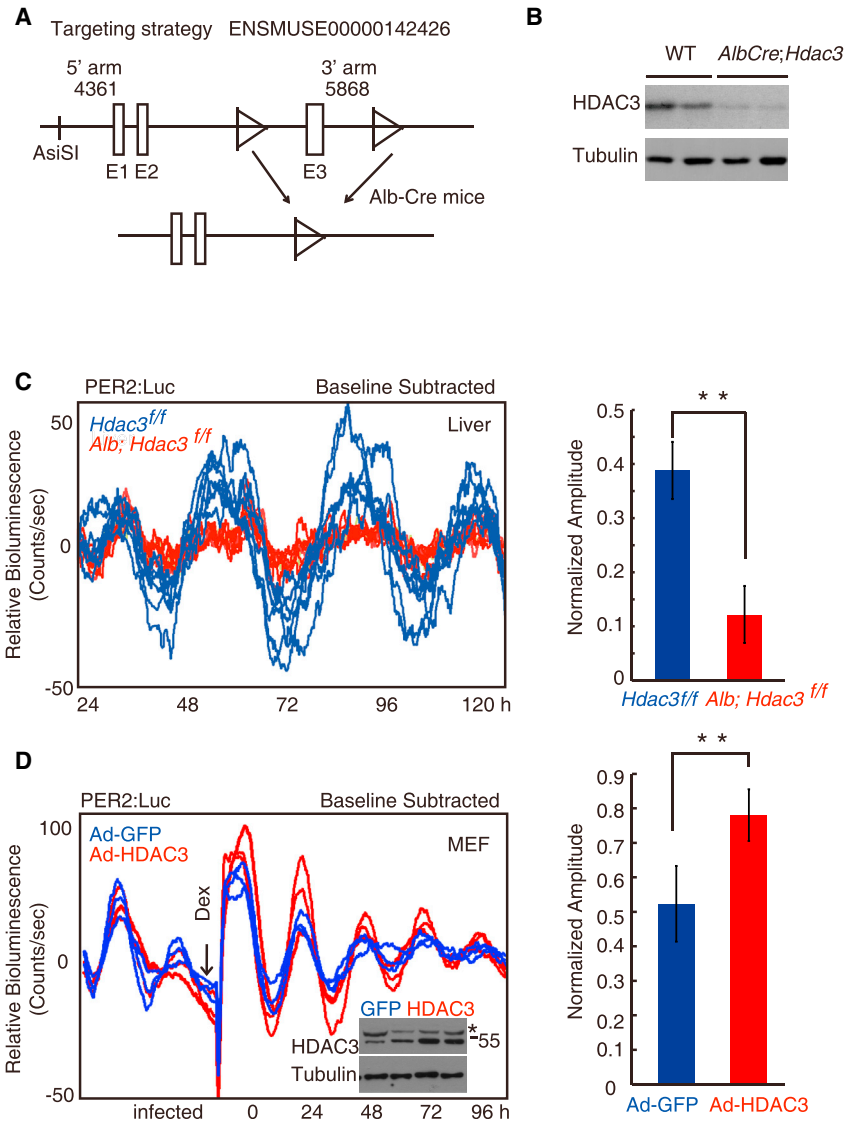



**Figure 1. HDAC3 Is Required for High-Amplitude Circadian Rhythms of *Per2*-Luciferase**

(A) Schematic of the targeting strategy for *Hdac3*-floxed mice. Targeted ESC colonies obtained from EUCOMM were used for blastocyst injections. The mice that retained the *loxP* sites flanking exon 3 at the endogenous *Hdac3* locus were bred to *Alb*-Cre mice in a C57BL/6N background to generate liver-specific knockout mice.

(B) Immunoblot with anti-HDAC3 antibody showing the depletion of the HDAC3 protein in *Alb; Hdac3*$^{f/f}$ liver tissues.

(C) Left: representative bioluminescence signals from liver slices of *Hdac3*$^{f/f}$; *Per2*$^{luc}$ (blue) and *Alb; Hdac3*$^{f/f}$; *Per2*$^{luc}$ (red) mice. Right: normalized amplitudes between cycles 2 and 4 were calculated as described in the Experimental Procedures. Means ± SD are shown (*Hdac3*$^{f/f}$; *Per2*$^{luc}$, 10 slices; *Alb; Hdac3*$^{f/f}$; *Per2*$^{luc}$, 12 slices from three independent experiments). **p < 0.001, Student's t test.

(D) Left: *Per2*$^{luc}$ MEFs were infected with either GFP-expressing (blue) or HDAC3-expressing (red) adenovirus for 60 hr and synchronized by a dexamethasone shock. The effect of *Hdac3* overexpression was determined using an immunoblot assay (bottom right). The asterisk indicates non-specific bands (only seen in MEFs but not in liver). Right: normalized amplitudes (n = 8 from three independent experiments) of the rhythms. **p < 0.001, Student's t test.

Despite their near-normal transcript levels, the BMAL1 and CRY1 protein levels were decreased markedly in *Alb*; *Hdac3*$^{f/f}$ livers (Figure 2B). In contrast to the accumulation of PER2 from CT 12–24 in WT mice, PER2 only accumulated at CT 16 and CT 20 in *Alb*; *Hdac3*$^{f/f}$ livers, but its rhythm was still robust. It should be noted that PER2 rhythmicity here is likely driven by systemic clues that can drive robust circadian rhythms in liver gene expression even when the hepatic molecular clock is disrupted (Kornmann et al., 2007).

We also obtained *Hdac1* and *Hdac2* conditional knockout mouse lines (*Hdac1*$^{tm1a(EUCOMM)Wtsi}$ and *Hdac2*$^{tm1a(EUCOMM)Wtsi}$) from the International Mouse Phenotyping Consortium to examine whether HDAC1 and HDAC2 have similar functions. These mice were crossed to *Alb-Cre* mice to obtain liver-specific knockout mice (*Alb; Hdac1*$^{f/f}$ and *Alb; Hdac2*$^{f/f}$ mice). As shown in Figures 2C and 2D, the genetic ablation of *Hdac1* or *Hdac2* did not have significant effects on the levels of BMAL1 and CRY1 in liver tissues as HDAC3 did, suggesting that these HDACs play different roles.

## HDAC3 Increases Enrichment of BMAL1 on E-boxes

The effect of HDAC3 on E-box-driven gene expression and on the abundance of BMAL1 prompted us to compare the enrichment of BMAL1 bound to *Dbp* E-box in the promoter (Ripperger and Schibler, 2006) and E-box of *Per2* genes (Yoo et al., 2005) in WT and *Alb*; *Hdac3*$^{f/f}$ livers. Chromatin immunoprecipitation (ChIP) assays revealed that the circadian rhythm of BMAL1 recruitment to E-boxes was impaired markedly in the *Alb*; *Hdac3*$^{f/f}$ liver (Figure 3A). Importantly, depletion of *Hdac3* only resulted in a reduction of BMAL1 association with E-boxes around the peak binding phases, when E-box-driven gene expression is maximally activated by the BMAL1-CLOCK complex (Figure 3A).

To determine whether HDAC3 is important for the recruitment of BMAL1 to E-box-driven genes, we infected MEFs with an adenovirus expressing HDAC3 or GFP for 48 hr and collected cells 15 and 24 hr after dexamethasone (DEX) treatment, which corresponded to the peak and trough time points of E-box transcriptional activity, respectively (Lee et al., 2008). ChIP assays using anti-BMAL1 antibody showed that HDAC3 expression dramatically enhanced the association of BMAL1 with the *Dbp* and *Per2* promoter E-boxes in the transcriptional activation

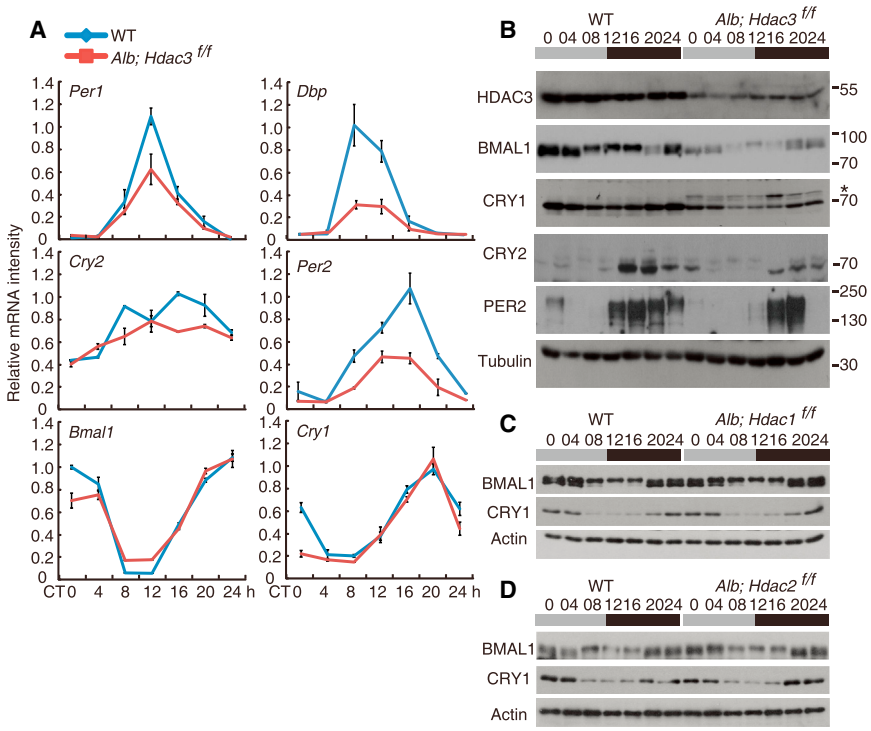



**Figure 2. mRNA and Protein Expression Levels of Clock Genes in WT and *Hdac3* Mutant Mice**

(A) The expression levels of the *Per1*, *Dbp*, *Per2*, *Cry1*, *Cry2*, and *Bmal1* genes in WT (*Hdac3*$^{f/f}$) (blue) and *Alb*; *Hdac3*$^{f/f}$ (red) liver tissues were measured by qRT-PCR and normalized to *Gapdh* expression. The expression levels were plotted as a.u., and the highest value in the sample was set to 1.0. Each value represents the mean ± SD (n = 3) from three mice.

(B) Protein profiles of HDAC3, BMAL1, CRY1, CRY2, and PER2 in total extracts from WT and *Alb*; *Hdac3*$^{f/f}$ liver tissues of 6- to 8-week-old mice. Tubulin was used as the loading control. Each image shows a representative example from three independent experiments. All of the tissues were collected at 4-hr intervals during the first day in constant darkness (DD). The samples and blots were processed in parallel in paired experiments. In this and all following figures, gray bars represent each subjective day, and the black bars represent subjective night. The extra protein band (indicated by an asterisk) above CRY1 is likely a non-specific band because it cannot be immunoprecipitated by an anti-CRY1 antibody.

(C and D) Circadian profiles of the BMAL1 and CRY1 proteins in WT, *Alb*; *Hdac1*$^{f/f}$ (C) and *Alb*; *Hdac2*$^{f/f}$ (D) liver tissues. Actin was used as the loading control.

phase (Dex 15) (Figure 3B). In contrast, HDAC3 expression had no effect at the Dex 24 time point (Figure 3B), indicating that the effect of HDAC3 on promoting BMAL1 recruitment is circadian-regulated and is mostly limited to the E-box transcriptional activation phase.

To examine whether the increased HDAC3-induced enrichment of BMAL1 contributes to the transcription of E-box-driven genes, we used DEX to entrain MEFs overexpressing Ad-GFP or Ad-HDAC3 for 48 hr. The expression levels of endogenous *Hdac3*, *Dbp*, and *Per2* were determined at various circadian time points. The overexpression of HDAC3 significantly increased the amplitudes of the circadian rhythms of the *Dbp* and *Per2* mRNAs, indicating that increased enrichment of BMAL1 to E-boxes by HDAC3 is important for the amplitude of transcript rhythms (Figure 3C). Surprisingly, expression of a deacetylase-dead HDAC3 mutant (H134Q/H135A/A136S) (Wen et al., 2003) had the same effects as WT HDAC3 (Figure 3C), indicating that this role of HDAC3 in the circadian clock is independent of its histone deacetylase function. Together, these results demonstrate that HDAC3 acts in the positive limb of the circadian negative feedback loop in a histone deacetylase-independent manner. HDAC3 functions by maintaining BMAL1 levels during the activation phase of E-box-driven circadian gene expression.

## HDAC3 Associates with BMAL1 and Promotes BMAL1 Ubiquitylation

To determine how BMAL1 is regulated by HDAC3, we examined whether BMAL1 and HDAC3 associate in cells. HEK293T cells were transfected with constructs expressing hemagglutinin (HA)-HDAC3 and FLAG-BMAL1. Immunoprecipitation assays showed the presence of HDAC3 in the anti-BMAL1 pull-down complexes (Figure 4A, top, lane 3). BMAL1 was also detected in the reciprocal pull-downs using anti-HA (HDAC3) antibody (Figure 4A, top, lane 6). No band was observed in the control samples.

Co-immunostaining of endogenous BMAL1 and HDAC3 in MEFs was also consistent with the interaction of HDAC3 with endogenous BMAL1 (Figure S2A). BMAL1 primarily localized to the nucleus. Although HDAC3 was found in both the nucleus and cytoplasm, it colocalized with BMAL1 in the nucleus (Figure S2A). Together, these results demonstrate that HDAC3 associates with BMAL1.

It has been suggested previously that BMAL1-CLOCK ubiquitylation is critical for the transcriptional activity of the complex (Lee et al., 2008; Stratmann et al., 2012). The reduced level of BMAL1 in *Alb*; *Hdac3*$^{f/f}$ liver tissue prompted us to examine the role of HDAC3 in this process. We found that HDAC3 expression markedly increased the amount of ubiquitylated BMAL1 (Figure 4B, lanes 2 and 6). The increase was even more pronounced when MG132 treatment was used to inhibit proteasome-mediated degradation (Figure 4B, lanes 4 and 8), suggesting that HDAC3 promotes BMAL1 ubiquitylation.

To confirm our observations in vivo, we compared the levels of ubiquitylated BMAL1 in the liver tissues of WT and *Alb*; *Hdac3*$^{f/f}$ mice at different circadian time points. As expected, the levels of ubiquitylated BMAL1 were reduced markedly in the *Alb*; *Hdac3*$^{f/f}$ liver (Figure 4C). Notably, the most pronounced decrease in ubiquitylated BMAL1 levels in *Alb*; *Hdac3*$^{f/f}$ mice occurred around the peak time points (CT 4–12) that correspond to the

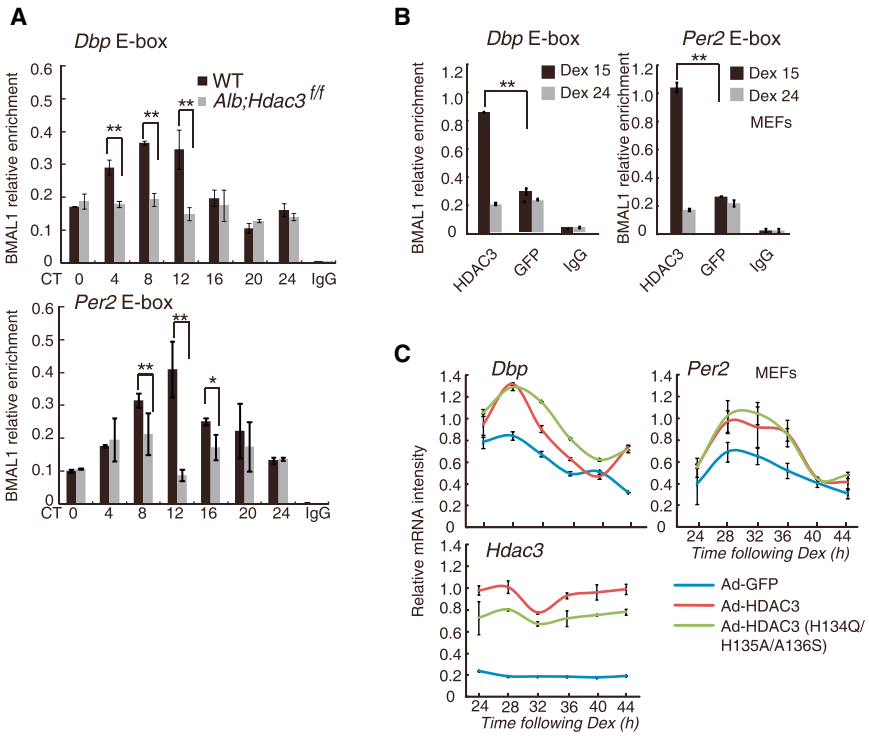



**Figure 3. HDAC3 Enhances the Enrichment of BMAL1 at the E-box during the Activation Phase and Increases the Amplitudes of *Dbp* and *Per2* mRNA Rhythms**

(A) BMAL1 binding to the E-box regions of the *Per2* and *Dbp* promoters during a circadian cycle (mean ± SD, n = 3) in WT (black) and *Alb; Hdac3*$^{f/f}$ (gray) livers. **p < 0.001, Student's t test. IgG served as an internal control. Chromatin samples from mouse livers were analyzed using ChIP with anti-BMAL1 antibodies. ChIP primers are described in Table S1.

(B) Synchronized WT MEFs were infected with adenoviruses overexpressing either GFP or HDAC3. Enrichment of BMAL1 at the *Per2* or *Dbp* promoters was detected by chromatin immunoprecipitation (IP) with anti-BMAL1 antibodies at the indicated time points (mean ± SD, n = 3; **p < 0.001, Student's t test). IgG, immunoglobulin G.

(C) The expression levels of *Dbp*, *Per2*, and *Hdac3* in WT MEFs infected with adenoviruses carrying GFP, HDAC3, or an HDAC3 mutant were measured by qRT-PCR and normalized to *Gapdh* expression. The MEFs were synchronized with a DEX shock and collected in a time course manner. The expression levels were plotted in a.u. Each value represents the mean ± SD (n = 3). Two-way ANOVA shows significant statistical differences ($p < 10^{-5}$) between GFP and HDAC3 or HDAC3 (H134Q/H135A/A136S) samples.

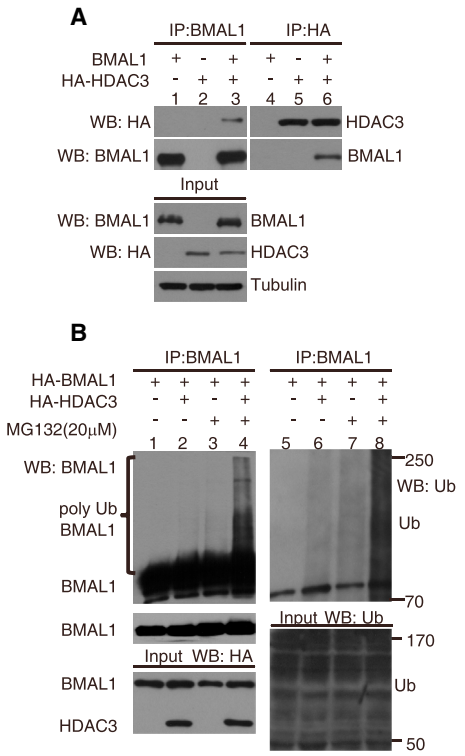
peak of BMAL1 binding to E-boxes (Figure 3A). These results indicate that, during the E-box activation phase, HDAC3 promotes BMAL1 ubiquitylation, which is known to be associated with the peak activity of the BMAL1-CLOCK complex (Kwon et al., 2006; Lee et al., 2008). Together, these results suggest that HDAC3 associates with BMAL1 and regulates the transcriptional activation activity of the BMAL1-CLOCK complex by promoting its ubiquitylation, which likely results in BMAL1 stabilization.

## HDAC3 Blocks FBXL3-Mediated CRY1 and CRY2 Degradation

The reduced CRY1 protein levels but near-normal *Cry1* mRNA levels in *Alb*; *Hdac3*$^{f/f}$ mice prompted us to examine the mechanism by which HDAC3 regulates CRY1 abundance. We first examined whether HDAC3 physically associates with CRY1 in cells. HEK293T cells were transfected with constructs expressing HA-HDAC3 and HA-CRY1 and were then immunoprecipitated with a CRY1 antibody. As shown in Figure 5A, little HDAC3 was detected in the CRY1 immunoprecipitates (Figure 5A, lane 1), suggesting that the association between HDAC3 and CRY1 is weak or transient. Surprisingly, when FBXL3 was co-expressed in these cells, abundant HDAC3 was found to be associated with CRY1 (Figure 5A, lane 2). FBXL3 is part of the E3 ligase that mediates the ubiquitylation and degradation of CRY1 (Busino et al., 2007; Siepka et al., 2007). The FBXL3-dependent increase in the HDAC3-CRY1 interaction occurred despite the decrease in CRY1 protein levels (Figure 5A, input lane 2). This effect of FBXL3 is dependent on its E3 ligase activity because expression of FBXL3 lacking its F-box (FBXL3$^{\Delta F\text{-box}}$: F-box deletion form) had no effect on the HDAC3-CRY1 interaction (Figure 5A, lane 3).

The ability of FBXL3 to promote an association between HDAC3 and CRY1 raises the possibility that HDAC3 may interact with FBXL3, a process that may affect CRY stability. HEK293 cells were transfected with constructs expressing FLAG-FBXL3 and HA-HDAC3. We found that FBXL3 coimmunoprecipitated with HDAC3 (Figure 5B). Consistent with this result, immunofluorescence analysis showed that FBXL3 and HDAC3 colocalized within the nucleus of HEK293T cells (Figure S2B). To examine the FBXL3 and HDAC3 interaction in vivo, we generated a bacterial artificial chromosome (BAC) transgenic mouse line encoding a FLAG and hemagglutinin (HA) N terminus-tagged *Fbxl3* gene (FH-FBXL3) (Figure 5C). The FH-FBXL3 BAC transgenic mice showed normal circadian rhythms (data not shown). The lysates of the FH-FBXL3 liver were then subjected to coimmunoprecipitation with a FLAG antibody. As shown in Figure 5C, HDAC3 was detected in the FLAG immunoprecipitates, indicating that HDAC3 and FBXL3 associate in vivo.

Because FBXL3 interacts with CRY1 to mediate its ubiquitylation and subsequent degradation, the association between HDAC3 and FBXL3 may, therefore, interfere in this process. To test this possibility, we compared the degradation rate of CRY1 in the presence of cycloheximide (CHX) to block de novo protein synthesis 24 hr after transfection of *Bmal1* and *Cry1*, *Fbxl3*, or *Hdac3* expression vectors in HEK293T cells. Similar to previous reports (Busino et al., 2007; Siepka et al., 2007), CRY1 underwent rapid degradation in the presence of FBXL3 (Figure 5D, center). However, the expression of HDAC3 almost completely blocked CRY1 degradation, even when



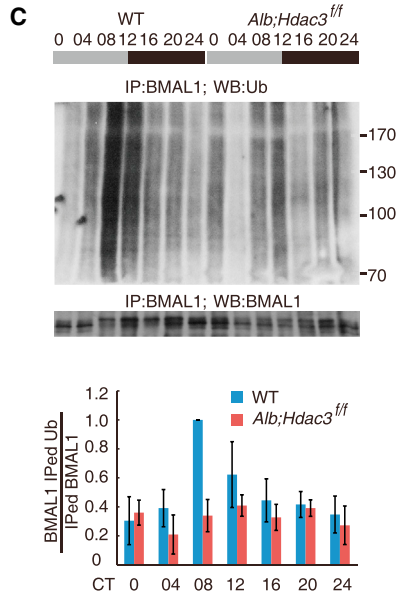




**Figure 4. HDAC3 Promotes the Ubiquitylation of BMAL1**

(A) The interaction between FLAG-BMAL1 and HA-HDAC3 was measured using IPs in HEK293T cells. Reciprocal IPs were performed with anti-BMAL1 (left) or anti-HA (right) antibodies. Subsequent detection of HDAC3 or BMAL1 was performed using anti-HA or anti-FLAG antibodies. WB, western blot.

(B) In vivo ubiquitylation assay of BMAL1. HA-HDAC3 and HA-BMAL1 were expressed in HEK293T cells. Twenty-four hours after transfection, the cells were treated with either 20 μM MG132 or DMSO (solvent) for 6 hr. The ubiquitylation of BMAL1 purified with anti-BMAL1 antibody was detected with antibodies against BMAL1 and ubiquitin (Ub).

(C) Liver lysates were collected at the different time points for each genotype and were then subjected to IP with an anti-BMAL1 antibody. Immunoprecipitates were blotted with an anti-ubiquitin antibody. BMAL1 purified with an anti-BMAL1 antibody was detected with an antibody against BMAL1. Bottom: the relative levels of ubiquitylated BMAL1 (quantified with NIH ImageJ software) in WT and *Alb; Hdac3*$^{f/f}$ liver tissues. Two-way ANOVA shows significant statistical differences between the two groups ($p < 10^{-4}$).

FBXL3 was overexpressed (Figure 5D, right). These results suggest that HDAC3 interferes with FBXL3-mediated CRY1 degradation.

To determine the mechanism by which HDAC3 interferes with CRY1 degradation, we expressed HA-CRY1, HA-HDAC3, FBXL3, or FBXL3$^{\Delta F\text{-box}}$ in HEK293T cells. Interestingly, coexpression of HDAC3 with CRY1 resulted in a marked accumulation of ubiquitylated CRY1 in the immunoprecipitate (Figure 5E, lanes 2 and 8). The level of immunoprecipitated, ubiquitylated CRY1 was increased further when FBXL3, but not FBXL3$^{\Delta F\text{-box}}$, was coexpressed (Figure 5E, lanes 4, 6, 10, and 12). In addition, HDAC3 and exposure to MG132 showed strong synergistic effects in promoting the accumulation of ubiquitylated CRY1 (Figure S3). Because the abundance of CRY2 protein was also decreased in *Alb*; *Hdac3*$^{f/f}$ mice, we performed parallel experiments with CRY2. We found that HDAC3 also promoted CRY2 ubiquitylation (Figure S4A) and protected FBXL3-mediated CRY2 degradation (Figure S4B). Taken together, these results suggest that HDAC3 blocks CRY protein degradation by interfering with the degradation of ubiquitylated CRY proteins.

## HDAC3 Promotes the Assembly of the BMAL1-CRY1 Complex during the Repression Phase

CRY transcriptionally represses the mammalian circadian negative feedback loop by interacting with the BMAL1-CLOCK complex (Lee et al., 2001; Yagita et al., 2002; Ye et al., 2014). The ability of HDAC3 to interact with BMAL1 and to associate with CRY1 in the presence of FBXL3 raises the possibility that HDAC3 and FBXL3 are involved in sequential assembly steps of the repressor complex in the circadian negative feedback process. To test this hypothesis, we transfected HEK293T cells with FLAG-tagged BMAL1, HA-tagged CRY1, and different combinations of HA-tagged HDAC3 and FBXL3. Very little BMAL1-CRY1 association was detected when *Bmal1* was coexpressed with *Cry1* (Figure 6A, lane 1). Remarkably, the expression of HDAC3, but not FBXL3, dramatically increased the BMAL1 and CRY1 association (Figure 6A, lane 4 versus lane 2). However, further addition of FBXL3 substantially enhanced the effect of HDAC3 on BMAL1-CRY1 association in an E3 ligase activity-dependent manner (Figure 6A, lane 5 versus lane 6), suggesting that ubiquitylation is important for the association. In contrast, the expression of FBXL21 (Hirano et al., 2013; Yoo et al., 2013) had no such effect (Figure 6A, lanes 7 and 8). These findings suggest that HDAC3 and FBXL3 promote association between BMAL1 and CRY1.

To confirm this conclusion in vivo, we examined the association between BMAL1 and CRY1 in the liver tissues of *Fbxl3* knockout and *Alb*; *Hdac3*$^{f/f}$ mice. As shown in Figure 6B, in the WT tissue, there was a robust rhythm of BMAL1-CRY1 association that peaked during subjective night, which corresponds to the transcriptional repression phase of E-box-driven genes. Although the levels of CRY1 and BMAL1 were similar at different time points in WT and *Fbxl3*$^{-/-}$ liver extracts, the association between BMAL1 and CRY1 was reduced significantly in *Fbxl3*$^{-/-}$ liver extracts (Figures 6B and 6C). Similarly, we found that the BMAL1-CRY1 association was reduced dramatically in *Alb*; *Hdac3*$^{f/f}$ liver extracts (Figures 6D and 6E).

Notably, in both the *Alb*; *Hdac3*$^{f/f}$ and the *Fbxl3*$^{-/-}$ liver extracts, the reduction in BMAL1-CRY1 association occurred primarily during subjective night, when CRY1 shows a peak in its

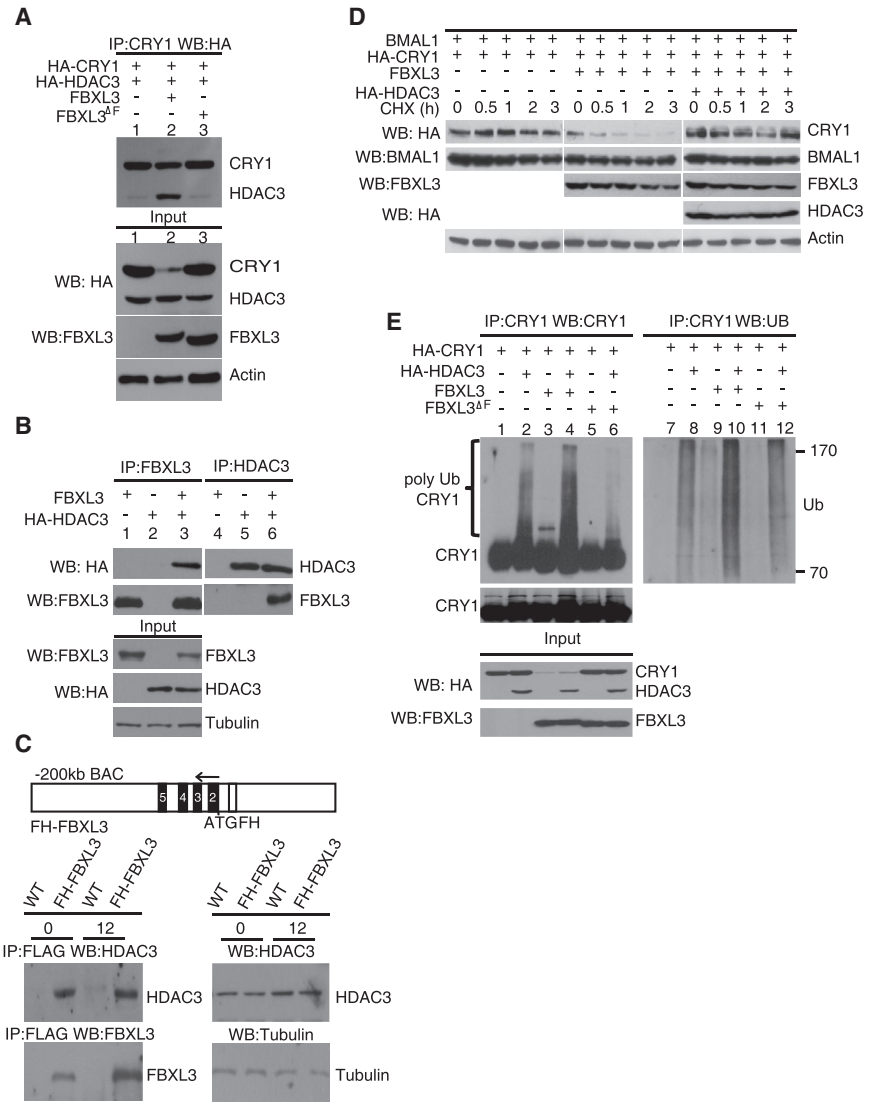



**Figure 5. HDAC3 Blocks FBXL3-Mediated CRY1 Degradation**

(A and B) The interactions between HDAC3 and CRY1 (A) and HDAC3 and FBXL3 (B) were detected using a similar strategy as in Figure 4A.

(C) The endogenous interaction between FBXL3 and HDAC3 was detected using an IP strategy with the indicated antibodies. The IPs were performed at two different time points (CT 0 and CT 12) using FH-FBXL3 BAC transgenic liver tissues. A schematic of the FH-FBXL3 BAC transgenic mice is shown at the top.

(D) The effects of HDAC3 on CRY1 stability. HA-CRY1, FBXL3, and BMAL1 were cotransfected with or without HA-HDAC3. Twenty-four hours after transfection, 20 μg/ml of CHX was added and incubated with the cells for the indicated time. Cell lysates were subjected to immunoblotting with the indicated antibodies, and actin was used as a loading control.

(E) In vivo ubiquitylation assay. HEK293T cells were transfected with HA-HDAC3, HA-CRY1, and FBXL3 or an F-box-deleted form of FBXL3 (ΔF-box). Twenty-four hours after transfection, the cells were lysed. MG132 treatment is shown in Figure S2. The ubiquitylation of CRY1 purified with anti-CRY1 antibody was detected with an anti-body against CRY1 and Ubiquitin.

interaction with and inhibition of the BMAL1-CLOCK complex. Together, these observations suggest that HDAC3 mediates the BMAL1-CRY1 interaction by blocking the degradation of CRY1 and promoting BMAL1-CRY1 interaction during the repressive phase, a process that inhibits the BMAL1-CLOCK complex. Therefore, HDAC3 has two seemly opposite roles in the circadian negative feedback loop: influencing both transcriptional activation and repression. The temporal separation of these two roles of HDAC3 ensures robust circadian gene expression.

## The Effect of HDAC3 on BMAL1 and CRY1 Is Independent of Its Deacetylase Activity and PER2

We found that mutations of HDAC3 (H134Q/H135A/H136S or S424A) that abolished its deacetylase activity (Wen et al., 2003; Zhang et al., 2005) did not affect its ability to interact with BMAL1, CRY1, or FBXL3 (Figures S5A–S5F) or to promote BMAL1 ubiquitylation (Figure 7A). In addition, treatment with the pan-HDAC inhibitor trichostatin A (TSA) also did not affect the ability of HDAC3 to promote the association between BMAL1 and CRY1 (Figure S6A). Furthermore, TSA treatment did not protect CRY1 from degradation (Figure S6B, 4). In contrast, overexpression of WT or catalytically dead HDAC3 was able to inhibit FBXL3-mediated CRY1 degradation (Figure S6B, 5 and 6) and to promote ubiquitylation of CRY1 (Figure S6C). More importantly, the overexpression of catalytically dead HDAC3 markedly increased the amplitude of *Per2*-luciferase oscillation in MEFs (Figure 7B). Together, these results indicate that the effect of HDAC3 on BMAL1 and CRY1 is independent of its deacetylase activity.

Similar to the roles of HDAC3 shown in this work, PER2 has also been shown to mediate the interaction between CRY1 and the BMAL1-CLOCK complex and to prevent CRY1 protein degradation (Chen et al., 2009; Yagita et al., 2002). To compare the role of PER2 and HDAC3, we expressed FLAG-tagged BMAL1, HA-tagged CRY1, and different combinations of HA-tagged HDAC3, PER2, and FBXL3 in HEK293T cells. BMAL1 immunoprecipitation showed that more CRY1 was immunoprecipitated with HDAC3 expression than with PER2 expression, despite the input level of CRY1 being higher in cells expressing PER2 than in cells expressing HDAC3 (Figure 7C, input lanes 3 and 7). Notably, additional FBXL3 expression did not enhance the association between BMAL1 and CRY1 when PER2 was expressed (Figure 7C, lane 8). This result is in contrast to the effect

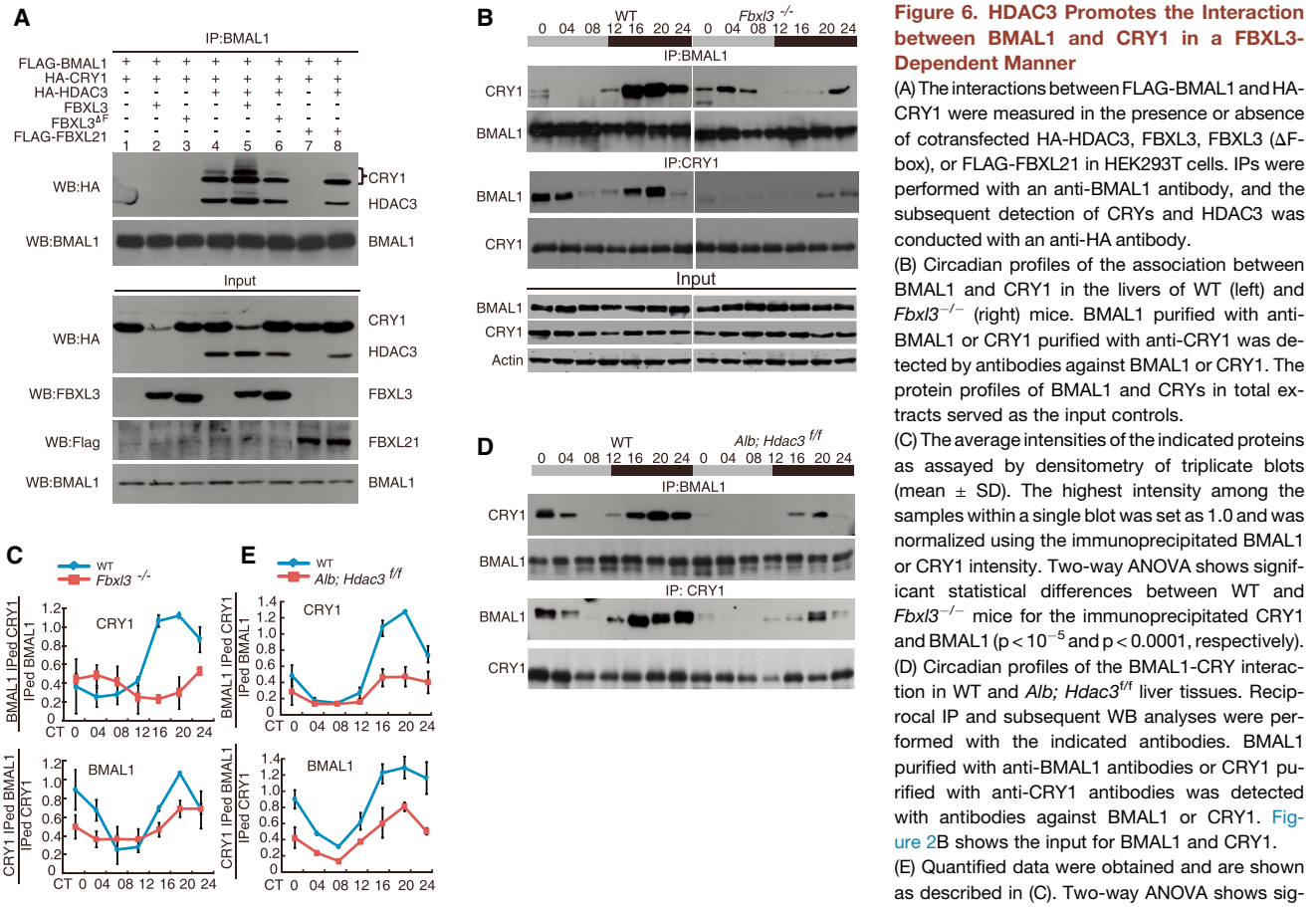



**Figure 6. HDAC3 Promotes the Interaction between BMAL1 and CRY1 in a FBXL3-Dependent Manner**

(A) The interactions between FLAG-BMAL1 and HA-CRY1 were measured in the presence or absence of cotransfected HA-HDAC3, FBXL3, FBXL3 (ΔF-box), or FLAG-FBXL21 in HEK293T cells. IPs were performed with an anti-BMAL1 antibody, and the subsequent detection of CRYs and HDAC3 was conducted with an anti-HA antibody.

(B) Circadian profiles of the association between BMAL1 and CRY1 in the livers of WT (left) and *Fbxl3*$^{-/-}$ (right) mice. BMAL1 purified with anti-BMAL1 or CRY1 purified with anti-CRY1 was detected by antibodies against BMAL1 or CRY1. The protein profiles of BMAL1 and CRYs in total extracts served as the input controls.

(C) The average intensities of the indicated proteins as assayed by densitometry of triplicate blots (mean ± SD). The highest intensity among the samples within a single blot was set as 1.0 and was normalized using the immunoprecipitated BMAL1 or CRY1 intensity. Two-way ANOVA shows significant statistical differences between WT and *Fbxl3*$^{-/-}$ mice for the immunoprecipitated CRY1 and BMAL1 ($p < 10^{-5}$ and $p < 0.0001$, respectively).

(D) Circadian profiles of the BMAL1-CRY interaction in WT and *Alb; Hdac3*$^{f/f}$ liver tissues. Reciprocal IP and subsequent WB analyses were performed with the indicated antibodies. BMAL1 purified with anti-BMAL1 antibodies or CRY1 purified with anti-CRY1 antibodies was detected with antibodies against BMAL1 or CRY1. Figure 2B shows the input for BMAL1 and CRY1.

(E) Quantified data were obtained and are shown as described in (C). Two-way ANOVA shows significant statistical differences between WT and *Alb; Hdac3*$^{f/f}$ mice for the immunoprecipitated CRY1 and BMAL1 ($p < 0.0001$ and $p < 0.001$, respectively).

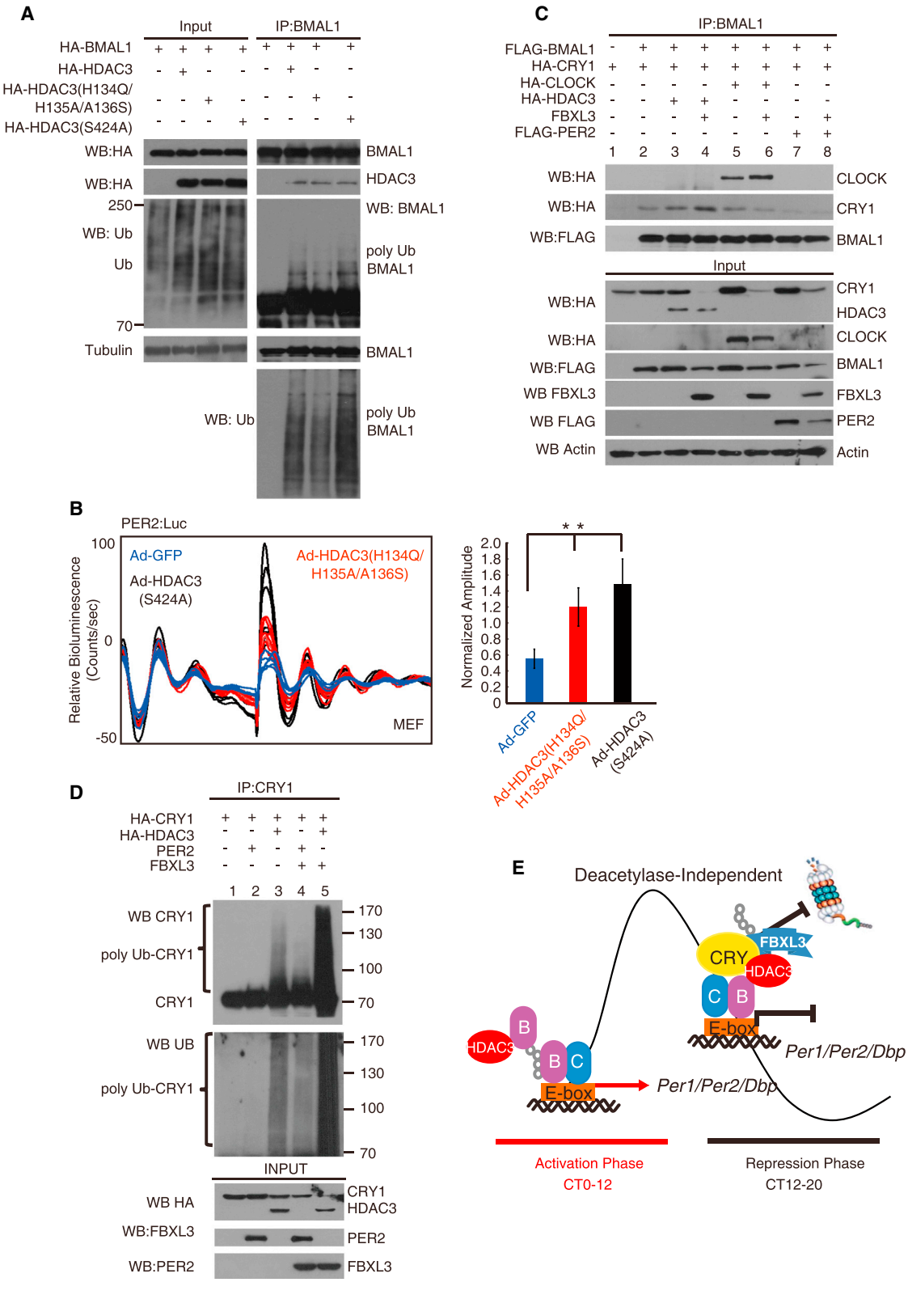
of FBXL3 when HDAC3 is expressed (Figure 6A). Furthermore, PER2 expression did not increase ubiquitylated CRY1 levels in the absence or presence of FBXL3 (Figure 7D). These results demonstrate that the role of HDAC3 in mediating the BMAL1-CRY1 interaction differs from and is independent of PER2. These results provide a likely mechanism for the ability of CRY1 to inhibit BMAL1-CLOCK-driven E-box transcription in the absence of PER proteins (Shearman et al., 2000; Ye et al., 2011, 2014).

## DISCUSSION

Cyclic activation and repression of E-box-driven transcription is the primary basis for circadian gene expression. In this study, we showed that liver-specific depletion of *Hdac3* led to low-amplitude rhythms and dampened E-box-driven transcription. In contrast, cellular overexpression of HDAC3 greatly increased the amplitude of *Per2*-luciferase rhythms. These results indicate that HDAC3 is critical for clock function.

Our results demonstrate HDAC3 is a critical component of the circadian negative feedback loop that regulates both transcriptional activation and repression processes in a deacetylase activity-independent manner. HDAC3 achieves these functions through two distinct mechanisms. During the activation phase, when E-box-driven transcription is maximally activated, HDAC3 promotes enrichment of BMAL1 at the E-box, likely by stabilizing BMAL1. In *Alb; Hdac3*$^{f/f}$ mouse liver tissues, the association of BMAL1 with E-boxes was reduced significantly from CT 8–12, explaining the reduced peak transcription levels of E-box-driven genes (Figure 3A). In contrast, overexpression of HDAC3 enhanced BMAL1 recruitment to the E-box (Figure 3B) and increased the peak levels of *Per2* and *Dbp* transcripts (Figure 3C).

The peak activity of the BMAL1-CLOCK complex is correlated tightly with BMAL1 ubiquitylation (Lee et al., 2008; Stratmann et al., 2012), suggesting that transcriptional activation by BMAL1 is coupled with its ubiquitylation. We found that HDAC3 associates with BMAL1 and that these proteins co-localize in the nucleus. Depletion of HDAC3 resulted in a marked reduction of BMAL1 ubiquitylation during the activation phase (Figure 4C). In contrast, overexpression of HDAC3 led to an increase in BMAL1 ubiquitylation. Although the presence of HDAC3 results in the accumulation of ubiquitylated BMAL1, depletion of *Hdac3* led to low BMAL1 levels (Figure 2B),

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suggesting that HDAC3 impairs the degradation of ubiquitylated BMAL1. Together, these results suggest that HDAC3 is required for efficient activation of E-box-driven transcription by promoting the accumulation of ubiquitylated BMAL1.

The rhythmic interaction between BMAL1 and CRYs, which peaks during subjective night, is essential for the negative feedback process (Czarna et al., 2011; Kiyohara et al., 2006; Lee et al., 2001; Sato et al., 2006). However, the mechanism of this interaction has not been clear (Lee et al., 2001). Here we show that HDAC3 plays a critical role in mediating the interaction between BMAL1 and CRY1. HDAC3 blocks FBXL3-mediated CRY1 degradation and strongly promotes BMAL1-CRY1 interaction, a process that inhibits BMAL1-CLOCK-dependent transcription during the repression phase (Figure 7E). Therefore, HDAC3 has two seemingly opposite functions in the circadian negative feedback loop: to enhance E-box-driven transcription and to repress the activity of BMAL1. However, these two functions of HDAC3 are separated temporally. The effect of HDAC3 on BMAL1 accumulation and on E-box-driven transcription occurs primarily during subjective day (CT 4–12), whereas its effect on the BMAL1-CRY interaction is primarily limited to subjective night (CT 16–24). Such a mechanism allows for maximum activation of E-box-driven transcription during the activation phase while also maximally repressing transcription during the repression phase, resulting in high-amplitude circadian rhythms.

The effect of HDAC3 on CRYs is mediated by FBXL3, the E3 ligase that mediates the ubiquitylation and degradation of CRY1 (Busino et al., 2007; Godinho et al., 2007; Siepka et al., 2007). FBXL3 interacts with HDAC3 and strongly promotes its association with CRYs. In addition, because HDAC3 blocks FBXL3-mediated CRY degradation, CRY levels are low when HDAC3 is depleted (Figure 2B). Therefore, both the stabilization of CRYs and the ability of HDAC3 to associate with both BMAL1 and CRYs can contribute the dramatic effect of HDAC3 on BMAL1-CRY association (Figure 7E). The mechanism for the temporal regulation of HDAC3 functions is currently unclear. Both BMAL1 and FBXL3 are subjected to rhythmic post-translational modifications. It is possible that such rhythmic modifications may affect the circadian phase-dependent functions of HDAC3.

HDAC3 has been shown previously to be recruited by REV-ERB$\alpha$ to direct rhythmic histone deacetylation (peaking at approximately CT 10), a process thought to be important for circadian output (Feng et al., 2011; Yin and Lazar, 2005). Such a role of HDAC3 is dependent on its histone deacetylase activity. However, the two roles of HDAC3 in the core circadian negative feedback loop are deacetylase activity-independent. Catalytically dead HDAC3 can still promote both E-box-driven transcription and the interaction between BMAL1 and CRYs. These results highlight the non-enzymatic role of HDAC3 in the circadian clock.

## EXPERIMENTAL PROCEDURES

### Animals

*Fbxl3*$^{-/-}$ mice have been described previously (Shi et al., 2013), and the *Alb-Cre* transgenic mice were obtained from The Jackson Laboratory (stock no. 003574). The *Per2*$^{Luc}$ mice were provided by Dr. Joseph Takahashi (University of Texas Southwestern Medical Center). To generate *Hdac3*-floxed mice, EUCOMM B6 embryonic stem cells (ESCs) were injected into blastocysts, and the resulting chimeric males were bred to C57BL/6J mice by following standard protocols. *Hdac1*- and *Hdac2*-floxed animals were obtained from the International Mouse Phenotyping Consortium (IMPC). For *FH-Fbxl3* mice, a BAC clone (bMQ186h21) was modified with a 3× FLAG-HA tag sequence inserted into the position corresponding to the N terminus of the *Fbxl3* coding region. Modified BACs were purified and injected into fertilized eggs to obtain BAC transgenic founders. All animal studies were carried out in an Association for Assessment and Accreditation of Laboratory Animal Care internationally accredited specific pathogen-free animal facility, and all animal protocols were approved by the Animal Care and Use Committee of the Model Animal Research Center, the host for the National Resource Center for Mutant Mice in China, Nanjing University.

### Luminescence Recording

The detailed methods for real-time measurement of luminescence from ex vivo tissues have been described previously (Liu et al., 2014; Yamazaki and Takahashi, 2005). For each genotype, 3–4 mice with 19–21 slices were examined. The MEFs from PER2::LUC mice (Yoo et al., 2004) were used at passages 3–4 and infected with recombinant adenovirus (GFP as a control, HDAC3, HDAC3 (H134Q/H135A/A136S), or HDACS424A) according to the experiment designs. 48–60 hr after infection, the cells were synchronized by DEX, and bioluminescence was recorded in real time with the LumiCycle (LumiCycle, Actimetrics). We previously developed a program (Liu et al., 2014) based on that of a method described previously (Izumo et al., 2006). This program allowed us to run the LumiCycle data to obtain a mean trend by a self-step algorithm. After subtracting the mean trend from the raw data and removing the damping effects, we used fast Fourier transform nonlinear least-squares (FFT-NLLS) to estimate the period and phase of the rhythms. The estimate of amplitude was then determined by a linear least-squares estimation of the detrended data. The normalized amplitude between cycles 2 and 4 was calculated by dividing the amplitude estimate by the average of luminescence intensity and was expressed as a fractional value.

**Figure 7. The Effect of HDAC3 on BMAL1 and CRY1 Is Independent of Its Deacetylase Activity and PER2**

(A) The interaction between HA-BMAL1 and HA-HDAC3 or the ubiquitylation of BMAL1 by HDAC3 was measured in the presence or absence of cotransfected HDAC3, HDAC3 (S424A), or HDAC3 (H134Q/H135A/A136S). IPs were performed with an anti-BMAL1 antibody, and subsequent detection of HDAC3 or BMAL1 was performed using anti-HA, anti-HDAC3, anti-BMAL1, or anti-ubiquitin antibodies as indicated.

(B) *Per2*$^{luc}$ MEFs were transfected with GFP-expressing (blue), mutant HDAC3 (S424A)-expressing (black), or HDAC3 (H134Q/H135A/A136S)-expressing (red) adenoviruses for 60 hr and synchronized with a DEX shock as described in Figure 1C. Right: normalized amplitudes (n = 8 from three independent experiments). $^{**}p < 0.001$, $^{*} < 0.05$.

(C) The interaction between FLAG-BMAL1 and HA-CRY1 was measured in the presence or absence of cotransfected HA-HDAC3, HA-CLOCK, FBXL3, or PER2 in HEK293T cells. The IPs were performed with an anti-BMAL1 antibody, and subsequent detection of CRY1, CLOCK, and HDAC3 was conducted with an anti-HA antibody.

(D) In vivo ubiquitylation assay of CRY1. HA-CRY1, HA-HDAC3, PER2, and FBXL3 were expressed in HEK293T cells. The cells were lysed 24 hr after transfection. The anti-CRY1 antibody immunoprecipitate was immunoblotted with anti-ubiquitin and anti-CRY1 antibodies.

(E) Model of the deacetylase-independent activity of HDAC3 with respect to the activation and repression phases of the circadian clock. B, BMAL1; C, CLOCK. The gray circle represents ubiquitylated BMAL1.

### Statistical Analysis

One-way or two way ANOVA followed by t test was performed using GraphPad Prism software to determine the significant differences between different genotypes. $p < 0.05$ was considered statistically significant.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.12.076.

## AUTHOR CONTRIBUTIONS

G.S., P.X., and Y.X. contributed to the experimental design, work, and data analysis. Z.Q., Z.Z., Z.D., L.X., Z.L., Y.A., and Y.D. contributed to the mouse work. G.X., L.Y., and Y.L. provided intellectual input. Y.L. assisted with the data analysis. G.S., Y.L., and Y.X. wrote the manuscript.

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